

**Amendments to the Specification**

**Page 1, Paragraph starting on Line 4**

This application claims priority to U.S. Provisional Application 60/252,744 filed November ~~November~~ 22, 2000 and U.S. Provisional Application 60/250,984 filed December 4, 2000, both of which are incorporated herein in their entirety.

**Page 1, Paragraph starting on Line 23**

CD 28 is one of the ~~principal~~<sup>T</sup> principal T cell costimulatory receptors. CD28 binds APC costimulatory ligands B7.1(CD80) and B7.2(CD86). CD28 is a transmembrane homodimer that is constitutively expressed on 90% of mammalian CD4+ T cells. Upon binding to the B7 family of molecules, CD28 delivers a powerful costimulatory signal for T cell activation and clonal expansion. Engagement of CD28 by its ligands B7-1 or B7-2 on the surface of APCs initiates a signaling cascade culminating in cytokine production and expansion of specific T-cells.

**Page 2, Paragraph starting on Line 4**

T cells also express cytotoxic-T-lymphocyte antigen 4, CTLA-4 (CD152), a close relative of CD28. In contrast to CD28, CTLA-4 is not expressed by naive T cells but is rapidly induced after T cell activation. Analogous to CD28, CTLA-4 also binds the B-7 family of molecules, albeit with higher avidity than CD28. Engagement of CTL4 with the B7 ligands transmits a negative signal to the T cells, thus terminating the immune response.

**Page 2, Paragraph starting on Line 10**

The critical role played by the B7/CD28:CTLA-4 costimulatory interaction in determining the fate of immune responses (activation vs anergy/apoptosis) makes it an attractive target for therapeutic immunomodulation in a wide range of autoimmune diseases. Recently, it has been shown that administration of monoclonal antibodies to B7 molecules and CTLA-4 Ig fusion protein ameliorate autoimmune diseases in various animal models, including (EAE) an animal model for MS, diabetes and systemic lupus erythematosus. However, these results depend

upon the timing of antibody administration. For example, CTLA-4 Ig was not effective in blocking already established EAE, and anti B7-2 mAB and anti CTLA-4 mAB exacerbate the disease. Moreover, the value of such antibodies as effective therapeutic agents is limited by ~~virture~~ virtue of their inherent immunogenicity and poor penetration across tissue ~~barriers~~ barriers.

**Page 3, Paragraph starting on Line 2**

The present invention provides new agents for blocking T cell-mediated immune reactions. Such agents are ~~peptides~~, peptides, referred to hereinafter as "CD28 peptide mimetics", of from 15 to 30 amino acids in length. The CD28 peptide mimetics comprise the hexapeptide motif 'MYPPPY' (SEQ ID NO: 1) or a retro-inverso isomer thereof. The CD 28 peptide mimetics further comprise flanking sequence at the amino and carboxyl terminus of the hexapeptide motif. Such flanking sequence permit the CD28 peptide mimetic to assume a polyproline II (PPII) conformation when placed in water or a buffered solution at physiological pH and a temperature of about 25° C.

**Page 3, Paragraph starting on Line 11**

The present invention relates to methods for treating subjects with T cell mediated autoimmune diseases or disorders. Such methods comprise administering one or more of the CD 28 peptide mimetics to a subject which has such disease or ~~disorder~~, disorder.

**Page 3, Paragraph starting on Line 20**

Figure 1: (A) CD spectra of end group blocked L CD28 (ELCD28) and retro-inverso CD28 peptide (RICD28) at 400μM in PBS, 50% TFE at 25° C and, (B) at 35° C. Mean residue ellipticity ( $\theta$ ) is expressed in degrees ~~cm<sup>2</sup>dmol<sup>-1</sup>~~ cm<sup>2</sup>dmol<sup>-1</sup>.

**Page 4, Paragraph starting on Line 27**

~~Figure 5:~~ Figure 5: Antigen-specific T-cell proliferative responses of lymph node cells and splenocytes from MBP peptide-specific TCR transgenic mice that carry a Vα4 Vβ 8.2 TCR treated with CD28 peptides. Single cell suspensions of CD4<sup>+</sup> T cells isolated from the (A)

lymph nodes and (B) spleen ( $5 \times 10^4$  cells /well) were stimulated with the encephalitogenic peptide of MBP, NAc 1-11 (10 $\mu$ g/ml) and cultured for a total of 72 hr (including an 18hr pulse with  $H^3$  thymidine) in the presence of varying concentrations of CD28 peptide analogues as shown. Data represents mean thymidine uptake and is plotted as delta cpm  $\pm$  SE. Results are mean of three different experiments. Proliferative responses of CD4 $^+$  LNC and spleen cells treated with L CD28, end group blocked CD28 and retro-inverso CD28 at all concentrations used were significantly less than untreated and control peptide (Reverse L CD 28 and D CD 28) treated cells \* = $p < 0.01$  by ANOVA.

**Page 5, Paragraph starting on Line 16**

Fig Figure 7: (A) CD28 synthetic peptide treatment increases apoptosis of CD4 $^+$  T cells ~~in-vitro~~ *in vitro*.  $5 \times 10^5$  CD4 $^+$  T cells were isolated from the pooled lymph nodes of V $\beta$ 8.2 V $\alpha$  4 TCR transgenic mice and stimulated with 10 $\mu$ g/ml of NAc 1-11 peptide of MBP either alone or in the presence of the specified concentrations CD28 APR. Lymph node cells were harvested after 48 hrs and apoptotic cells among V $\beta$ 8.2 $^+$  cells were detected by the TUNEL methods and analyzed by flow cytometry. The data shown is the average results from three experiments. Significant increase in the percentage of apoptotic cells was observed following treatment with 150 $\mu$ M EL CD28 and 75 $\mu$ M ~~or 150 $\mu$ M~~ or 150 $\mu$ M RI CD28 APR. Lymph node cells alone or when treated with the CD28 peptides in the absence of antigen showed maximum apoptosis (Data not shown). \* =  $p < 0.01$  by one way ANOVA. Representative histograms showing increase in FITC conjugated TUNEL positive cells in cultures treated with 120 $\mu$ M EL CD28 or RI CD28 are shown in (B) and (C) ~~respectively~~ respectively.

**Page 5, Paragraph starting on Line 30**

~~Fig-8.~~ Figure 8: Injection of synthetic CD28 peptide analogues inhibits development of clinical EAE and attenuates established EAE. B10.PL mice were immunized with GP-MBP in CFA and injected pertussis ~~toxin i.p.~~ toxin i.p. on day 0 and 2. The data are presented as the mean clinical score in each group on different days of observation. Animals received a single i.v. injection of 500 ~~µg~~  $\mu$ g of ELCD28(n=10) or RI CD28(n=10) peptides or control [LCD28(n=12), RLCD28 (n=6) and D CD28(n=6)] peptides or PBS (n=12) on the day of immunization (A) or on

day 14 post-immunization (B). Data represent the means of pooled data from two separate experiments.

**Page 6, Paragraph starting on Line 11**

The CD 28 peptide mimetics are from 15 to 30 amino acids, preferably from 17 to 25 amino acids, more preferably from 19 to 21 amino acids in length. The CD28 peptide mimetics comprise a hexapeptide with the following sequence: MYPPPY, ~~SEQ ID NO: 1~~ SEQ ID NO: 1, or the retro-inverso isomer thereof, i.e., YPPPYM, ~~SEQ ID NO: 2~~ SEQ ID NO: 2. The CD28 peptide mimetics further comprise flanking sequences, i.e. a plurality of amino acids, at the amino and a plurality of amino acids at carboxy termini of the hexapeptide motif. Preferably, the hexapeptide is sandwiched between two ~~amphiphilie~~ amphiphilic, anti-parallel right-twisted B strands. For optimum stability of the CD28 peptide ~~mimetic~~ mimetic, it is preferred that the antiparallel B strands be of the same ~~length~~ length.

**Page 6, Paragraph starting on Line 21**

In those instances where the methionine is at the amino terminus of the hexapeptide, the CD 28 peptide mimetic, referred to hereinafter as "L" peptide mimetic, is comprised of levorotary amino acids. In those instances where the methionine is at the carboxy terminus of the hexapeptide, the CD 28 peptide mimetic, referred to hereinafter as a "D" peptide mimetic, is comprised of ~~dexorotary~~ dextrorotary amino acids such that the D peptide mimetic is a topochemical equivalent of the corresponding L peptide mimetic. The retro-inverso modification of the L-peptide mimetic to produce a corresponding D peptide mimetic involves the reversal of all amide bonds within the peptide backbone. This is achieved by reversing the direction of sequence and inverting the chirality of each amino acid residue by using D-amino acids. The goal of this topochemical approach is to create an analog such that the reversed amide bonds in the D peptide mimetic retains both the planarity and conformational restrictions of peptide bonds (CONH) and the spatial orientation of side chains remains closely related to that of the corresponding L peptide mimetic. Advantageously, the D peptide mimetic is resistant to proteases that are present in mammals.

**Page 7, Paragraph starting on Line 3**

The amino and carboxy termini of the CD 28 peptide mimetics may be free or, preferably, end-blocked. When placed in water or a buffered solution having a pH of about 7.4, the CD 28 peptide mimetics adopt a PPII helical conformation. The secondary structure of the CD 28 peptide and the presence of a PPII helical conformation may be determined using a circular ~~dichromism~~ dichroism assay.

**Page 7, Paragraph starting on Line 8**

The CD 28 peptide mimetics bind B7 molecules on APC's (Antigen Presenting Cells) with an affinity which, preferably is less than the affinity of CTLA-4 for these molecules. The CD 28 peptide mimetics bind B7 molecules with an affinity which is equivalent to the affinity of CD28 for these ligands. This property can be determined empirically using a ~~competitive~~ competitive binding ~~analysis~~ analysis. Alternatively, the relative affinity of the CD 28 peptide mimetic for B7 molecules can be estimated on the basis of ~~K<sub>a</sub>~~ K<sub>a</sub> and ~~K<sub>d</sub>~~ K<sub>d</sub> measurements.

**Page 7, Paragraph starting on Line 15**

The CD 28 peptide mimetic has a ~~K<sub>d</sub>~~ K<sub>d</sub> which is equivalent to the ~~K<sub>d</sub>~~ K<sub>d</sub> of CD28. The CD 28 peptide mimetic has a ~~K<sub>d</sub>~~ K<sub>d</sub>, preferably, between 2 and 3 micromoles, more preferably, between 2.1 and 2.7 micromoles. Thus, the CD 28 peptide mimetic binds to the B71 ligand and the B7 2 ligand, which are also known as CD 80 and CD 86 respectively, with fast kinetics.

**Parge 7, Paragraph starting on Line 20**

In certain embodiments, the L form of the CD28 peptide mimetic comprises the following sequence: FMYPPPYL, ~~SEQ ID NO: 3~~ SEQ ID NO: 3. The corresponding D form of this peptide mimetic is the retro inverso isomer of this sequence. In certain embodiments, the D form of the CD 28 peptide mimetic comprises the sequence LYPPPYMFEIK, ~~SEQ ID NO: 4~~ SEQ ID NO: 4. In one embodiment, the CD28 peptide mimetic is an L-peptide which comprises 20 L-amino acids, has the sequence KIEFMYPPPYLDNERSNGIE, ~~SEQ ID NO: 5~~ SEQ ID NO: 5, and has free ends. In another embodiment, the peptide mimetic is an L-peptide which comprises 20 L-amino acids, has the sequence KIEFMYPPPYLDNERSNGIE, ~~SEQ ID NO: 5~~ SEQ ID NO: 5 and has blocked ends; i.e., the lysine at the amino terminus is acetylated and the

glutamic acid at the carboxy terminus is amidated. In a further embodiment, the peptide mimetic is a D-peptide which comprises 20 D-amino acids and has the sequence EIGNSRENDLYPPPYMFIEK, ~~SEQ ID NO: 6~~ SEQ ID NO: 6, and has free ends. In another embodiment, the peptide mimetic is a D-peptide which comprises 20 D-amino acids and has the sequence EIGNSRENDLYPPPYMFIEK, ~~SEQ ID NO: 6~~ SEQ ID NO: 6, wherein the aspartic acid residue at the amino terminus is acetylated and the lysine residue at the carboxy terminus is amidated. In certain embodiments of the L form of the CD28 peptide mimetic, flanking regions of the core hexapeptide comprise a repetitive LS sequence; while the flanking regions of the corresponding D form of the CD28 peptide mimetic comprise a repetitive SL sequence. Thus, the CD 28 peptide mimetic may comprise one of the following sequences

LSLSLSMYPPPYLSLSLS, ~~SEQ ID NO: 7~~ SEQ ID NO: 7,

LSLSLSKEIFMYPPPYLDNESLSLSLS, ~~SEQ ID NO: 8~~ SEQ ID NO: 8,

SLSLSIYPPPYMSLSLSLS ~~SEQ ID NO: 9~~ SLSLSLYPPPYMSLSLSLS SEQ ID NO: 9,

and

SLSLSENDLYPPPYMFIEKSLSLSL, ~~SEQ ID NO: 10~~ SEQ ID NO: 10.

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The present CD 28 peptide mimetics also encompass peptides that are biologically equivalent variants of ~~SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10.~~ SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10. A “biologically equivalent variant” as used herein, refers to a peptide whose amino acid sequence is similar but not identical to the amino acid sequence of one of these sequences, hereinafter referred to as the “reference” amino acid sequence, but does not have 100% identity with such reference sequence. Peptides which are biologically equivalent variants have an altered sequence in which one or more of the amino acids in the reference sequence other than the hexapeptide motif, i.e., in the flanking sequence, is substituted, or in which one or more amino acids are deleted from or added to one or both of the flanking

sequences of the hexapeptide motif. Preferably the deletions and additions are located at the amino terminus, the carboxy terminus, or both, of one of the sequences shown above.

**Page 9, Paragraph starting on Line 11**

Peptides which are biologically equivalent variants of CD 28 peptide mimetics comprising ~~SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10~~ SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10 bind to B71 and B72 with an affinity that is less than CTLA-4 and comparable to CD28. Peptides which are biologically equivalent variants of CD 28 peptide mimetics comprising ~~SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9~~ SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9 have a ~~K<sub>d</sub>~~ K<sub>d</sub> which is between 2 and 3 micromoles.

**Page 9, Paragraph starting on Line 18**

The present invention also provides a method of treating the symptoms of a disease or disorder that involves a deleterious activation of T cells. Examples of such diseases are autoimmune diseases, such as MS, EAE which is the mouse model for MS, rheumatoid arthritis, and insulin-dependent diabetes mellitus. One example of another disorder that involves deleterious activation of T cells is rejection of an allograft ~~transplant~~ transplant. Such method comprises administering a pharmaceutical composition, which comprises an L CD 28 peptide mimetic, a D CD 28 peptide mimetic or both to a subject in need of the same. As used herein, the term subject refers to a mammalian animal, preferably a human. By "treating" is meant ameliorating or tempering the severity of the disorder or the symptoms associated therewith. In such cases, as for example multiple sclerosis, the pharmaceutical composition is administered either when patients have clinical symptoms, or when a genetic mutation indicative of MS is identified. Preferably, the protocol involves oral administration of a pill or water-soluble mixture, or injection, preferably intravenous injection, of the pharmaceutical composition. In the case of rheumatoid arthritis, the pharmaceutical composition may be administered when patients exhibit clinical symptoms of the disease. In the case of insulin-induced diabetes mellitus, the pharmaceutical composition is administered when patients have clinical symptoms, or when a

genetic mutation indicative of diabetes mellitus is identified. The protocol involves oral administration of the pharmaceutical composition, which preferably is in the form of a pill or water soluble mixture, or injection of the pharmaceutical composition, preferably intravenous injection.

**Page 10, Paragraph starting on Line 8**

The present invention also relates to a method of preventing T-cell mediated rejection of an allograft transplant. The method comprises administering a CD 28 peptide ~~mimete~~ mimetic to a patient that has recently undergone, or is about to undergo, such transplant. Preferably, the peptide mimetic is administered to such a patient intravenously.

**Page 10, Paragraph starting on Line 14**

The CD 28 peptide mimetics are prepared using standard techniques and equipment for preparing synthetic peptides, such as a synthesizer. For example, the CD 28 peptide mimetics may be prepared using the 9600 Millegen/Biosearch synthesizer or a 40 well multiple peptide synthesizer (MPS 396, Advanced Chem Tech, Louisville, KY) and purified by reverse phase HPLC (Water's Associates) and characterized by electrospray ionization spectrometry (Mass Spectral facility, OSU). Retro-inverso peptides are assembled in a reverse order of amino acids with Fmoc-D-aminoacid amino acid derivatives.

**Page 11, Paragraph starting on Line 5**

~~In-vivo~~ In vivo, a biologically effective amount is an amount sufficient to sufficient to show a meaningful benefit, i.e., partially or completely relieve the symptoms associated with the respective disease or disorder. The amount of the CD 28 peptide mimetic required will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the subject has undergone and the type of defect or disease being targeted. Ultimately, the dosage will be determined using clinical trials. Initially, the clinician will administer doses that have been derived from animal studies. An effective amount can be achieved by one administration of the composition. Alternatively, an effective amount is achieved by multiple



administration of the composition to the subject. ~~In vitro~~ In vitro, the biologically effective amount is the amount sufficient to reduce proliferation or activation of CTLA4+ T cells.

**Page 11, Paragraph starting on Line 24**

MS is a demyelinating disease of the central nervous system that likely results from a combination of genetic susceptibility, environmental factors, pro-inflammatory cytokines released in the CNS and autoimmune reactions. ~~MS reactions.~~ MS pathology consists of CNS inflammatory infiltrates containing CD4+ autoreactive T cells and demyelination. Hence, MS is considered to be an autoimmune disease of the CNS that is triggered by an unknown stimulus. A cascade of inflammatory events leads to the activation of the immune system that perpetuates the inflammatory process. T cells specific for myelin antigens arise as a primary or secondary event in MS, which damages myelin and destroys oligodendrocytes leading to demyelination. Thus a CD4+ T cell mediated immune response plays a central role in the pathogenesis of MS. Depending on the extent of demyelination and rate of remyelination, the MS disease course is variable and is classified as relapsing-remitting, primary progressive, secondary progressive and progressive relapsing.

**Page 12, Paragraph starting on Line 4**

EAE is an animal model used to design and test interventions in the MS disease process because of its clinical, immunological and histopathological similarities to MS. EAE is an experimental autoimmune disease of the CNS that results from the immunization of susceptible animals with myelin proteins, including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) or peptides derived from these proteins. EAE is mediated by activated ~~CD4+T cells~~ CD4+ T cells that recognize the neuroantigen in the context of major histocompatibility complex (MHC) Class II molecules. The resulting immune response initiates a series of events including CNS mononuclear cell infiltration, demyelination, perivascular edema, and ascending paralysis. EAE in susceptible mouse strains, including B10.PL, SLJ, and PL/J is characterized by a relapsing remitting (R-EAE) disease course, in which animals undergo an initial acute episode, followed by remission, with progression to multiple relapses. R-EAE is initiated in mice by either immunization with myelin antigens

combined with CFA and pertussis toxin (PT) or by adoptive transfer of activated ~~myelin specific~~ myelin specific CD4<sup>+</sup> T cells. The N acetylated 1-11 (NAc 1-11) peptide of MBP is the immunodominant epitope recognized by encephalitogenic CD4<sup>+</sup> T cells in the context of MHC Class II I-A<sup>v</sup> (H-2<sup>v</sup>) in the B10.PL mouse. In the immune response to this peptide in I-A<sup>v</sup> mice, there is preferential usage of the Vb8 T cell receptor (TCR) genes. This finding has led to the development of TCR transgenic mice overexpressing the MBP-specific TCR (~~Va4 V~~ (Va4 V)).

**Page 13, Paragraph starting on Line 18**

Based on molecular modeling of the CD28 extracellular domain, a 20 residue linear peptide was defined that comprised the conserved polyproline motif and flanking sequence such that the predicted sequence had a greater propensity to form an helical structure as predicted by the secondary structure algorithm by Chou and Fasman. The sequence of this L form of the CD 28 peptide mimetic is KIEFMYPPTYLDNERSNGIE, ~~SEQ ID NO: 5~~ SEQ ID NO: 5.

**Page 13, Paragraph starting on Line 24**

In order to mimic the end groups of the ligand binding epitope of parent CD28 molecule the amino terminus of free L CD28 peptide mimetic was acetylated and the carboxy terminus was amidated. In addition, this modification neutralizes charges at the termini of the peptide. This modification stabilizes the secondary ~~stureture~~ structure, and is expected to enhance the functional interaction of the molecule with B7 ligands.

**Page 13, Paragraph starting on Line 29**

Unmodified peptides can be susceptible to enzymatic degradation and rapid clearance from circulation. Accordingly, a retro-inverso isomer of the above described L CD28 peptide mimetic was designed. Retro-inverso peptides are peptides made of reversed D-amino acids, so they are mirror images of a mirror image. The use of D amino acids results in inverted chirality and the reversed order of amide bonds (-NHCO- instead of -CONH-) and creates an analogue that regenerates both the planarity of peptide bonds and the spatial orientation of side chains closely related to that of the original peptide. The retro-inverso peptide was assembled in a reverse order of amino acids with Fmoc-D-aminoacid amino acid derivatives. The retro-inverso

peptide, i.e., the D CD28 peptide has the sequence EIGNSRENDLYPPPYMFIEK, ~~SEQ ID NO: 6~~ SEQ ID NO: 6.

**Page 14, Table 1**

CD28 Peptide Sequence	<u>SEQ ID NO:</u>	Abbreviation	Identity
<b>NH<sub>2</sub>KIEFMYPPPYLDNERSNGTICOOH</b>	<u>SEQ ID NO: 11</u>	L-CD28	<i>Free L peptide</i>
CH <sub>3</sub> COL[KIEFMYPPPYLDNERSNGTI]LCONH <sub>2</sub>	<u>SEQ ID NO: 11</u>	EL-CD28	<i>End-blocked L-peptide</i>
CH <sub>3</sub> COD[ITGNSRENDLYPPPYMFIEK]DCONH <sub>2</sub>	<u>SEQ ID NO: 12</u>	RI CD28	<i>Retro-inverso D-peptide</i>
CH <sub>3</sub> COD[KIEFMYPPPYLDNERSNGTI]DCONH <sub>2</sub>	<u>SEQ ID NO: 11</u>	D-CD28	<i>D-peptide (Control)</i>
CH <sub>3</sub> COL[ITGNSRENDLYPPPYMFIEK]LCONH <sub>2</sub>	<u>SEQ ID NO: 12</u>	RL-CD28	<i>Reverse-L-peptide (Control)</i>

**Page 14, Paragraph starting on Line 14**

The CD 28 peptide mimetics and control ~~petides~~ peptides shown in Table 1 above were synthesized by solid phase peptide synthesis following Fmoc/DCC/HOBt methodology on a fully automated peptide synthesizer (Model 396-5000 Multiple Peptide Synthesizer, Advanced Chemtech, Louisville, KY). The free L CD28 peptide was assembled on 4-methylbenzhydrylamine resin (0.5mmol/g substitution) with 4-(hydroxymethyl) phenoxyacetic acid as the linker. The end group blocked peptides were assembled on Fmoc-2, 4-dimethoxy-4'- (carboxymethyloxy)-benzylhydramine (Rink amide) resin (Advanced Chemtech, Louisville, KY) as peptide amides. Coupling reactions utilized six equivalents of each amino acid with 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3, -tetramethyluronium tetrafluoroborate and distilled diisopropylethylamine (DIEA). Deprotection was accomplished with 30% piperidine in DMF. Immediately after the final deprotection step, the free NH<sub>2</sub> group of the terminal amino acid residue was acetylated with 5ml of 3mmol

acetimidazole in DMF (50 ml). The completion of acetylation was confirmed by a negative Kaiser Ninhydrin test. With respect to the parent peptide, the retro-inverso peptide was assembled in reverse order of amino acids with Fmoc-D-amino acid derivatives. The peptides were cleaved from the resin support with simultaneous side-chain deprotection by acidolysis using TFA with 5% phenol, 5% thioanisole and 2.5% ethanedithiol as scavengers. The crude peptides were purified by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) using a C<sub>18</sub> column (10mm by 25cm) (Vydac, Hesperia, CA) at a temperature of 32.5° C and a flow rate of 5ml/min. Peptides (5-10mg per run) were loaded in 0.1M acetic acid and chromatographed for 30 min with a linear gradient of 10-60% of acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). The separations were monitored at 230 and 280 nm.

**Page 15, Paragraph starting on Line 12**

Analytical HPLC was run using a VIDAC C<sub>18</sub> column (4.6 mm by 25 cm) using the same gradient as stated above. Eluants were monitored at 214 and 254 nm. Purified ~~peptides were~~ peptides were obtained in greater than 95% purity as assessed by reverse-phase HPLC. The identity of peptides was finally confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry.

**Page 15, Heading starting on Line 18**

**A. ~~Sturctural~~ Structural Characterization of the Synthetic CD28 Peptide Mimetics and Control Peptides**

**Page 17, Paragraph starting on Line 6**

The CD spectrum of the RI CD28 peptide mimetic at 25° C presented a mean residue ellipticity maximum at 205nm ( $\theta = 57 \times 10^3 \text{ deg cm}^2 \cdot \text{dmol}^{-1}$ ), a weak minimum at 215 nm ( $\theta = 1.14 \times 10^4 \text{ deg cm}^2 \cdot \text{dmol}^{-1}$ ) and a weak maximum at 223 nm ( $\theta = 18.3 \times 10^3 \text{ deg cm}^2 \cdot \text{dmol}^{-1}$ ) (Fig 1A). Similar mirror-image like CD spectra of retro-inverso isomers of L peptides have been previously reported (Petit, M. C., N. Benkirane, G. Guichard, A. P. Du, M. Marraud, M. T. Cung, J. P. Briand, and S. Muller. 1999. Solution structure of a retro-inverso peptide analogue

mimicking the foot-and-mouth disease virus major antigenic site. Structural basis for its antigenic cross-reactivity with the parent peptide. *J Biol Chem* 274:3686.) By increasing the temperature to 90<sup>0</sup> C, the molar ellipticity maximum markedly decreased in intensity ( $\theta = 17.2.9 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ ) and shifted to a longer wavelength (211nm). The CD spectrum of the retro-inverso CD28 peptide mimetic dissolved in 6M CaCl<sub>2</sub> showed a dramatic decrease in molar ellipticity maximum at 205nm ( $\theta = 1.10 \times 10^4 \text{ deg cm}^2 \text{ dmol}^{-1}$ ), complete loss of molar ellipticity ~~minimumat~~ minimum at 215nm and the maximum at 223nm (Fig 1B). These observations reflect the ~~destabilizingeffect~~ destabilizing effect of CaCl<sub>2</sub> on the PPII helical conformation adopted by the retro-inverso CD28 peptide mimetic.

**Page 17, Paragraph starting on Line 23**

The CD spectrum of a six residue free peptide comprising the "MYPPPY" (SEQ ID NO: 1) motif alone showed a weak minimum ( $\theta = -8.6 \times 10^1 \text{ deg cm}^2 \text{ dmol}^{-1}$  cm<sup>2</sup> dmol<sup>-1</sup>) at 208 nm (data not shown). This suggests that the length of the CD28 peptide and the side chain interactions with the flanking residues play a role in the formation of a PP II helix.

**Page 17, Paragraph starting on Line 29**

Binding experiments were performed by surface plasmon resonance (SPR) on a ~~BIAcore~~ an instrument sold under the trademark BIACORE™ from Pharmacia Biosensor (Uppsala, Sweden). All experiments were performed at 37<sup>0</sup> C using HBS-EP buffer (25mM Hepes, pH7.4, 150nM NaCl, 3.4mM EDTA and, 005% surfactant P20) supplied by Pharmacia Biosensor.

**Page 18, Paragraph starting on Line 30**

Data analysis was performed with ~~BIAevaluation~~ BIAEVALUATION™ software version 2.1(Pharmacia Biosensor AB). The binding as measured in response units (RU) in ~~BIAcore~~ BIACORE™ and the binding rate,  $dR/dt$ , can be used to evaluate the kinetics of the synthetic CD28 peptides - CD80-Ig interaction. Prior to kinetic analysis, data were adjusted to zero baseline level by subtracting the background responses obtained by injection of the analytes through a control flow cell with no ligand immobilized. Data from direct kinetic analysis were

analyzed as follows. First, the dissociation rate constant,  $k_d$ , (units:  $s^{-1}$ ) was determined, by fitting experimental data from the buffer flow part of the sensogram to the equation

$$R(t) = R_1 \cdot e^{-k_d(t-t_1)} \dots\dots\dots(1)$$

where  $t_0$  is the injection time,  $R_1$  is the response level at the start of dissociation time  $t_1$  and  $k_d$  is the dissociation rate constant.

**Page 19, Paragraph starting on Line 9**

The  $k_d$  value obtained was used as a constant during the analysis of the injection phase data. Binding data above the noise level was selected by converting the ~~sensogram~~ sensogram to a plot of the logarithm of the binding rate vs time ( $dR/dt$  vs. time). The  $k_a$  was determined by nonlinear curve fitting of the following equation

$$R(t) = R_{eq} * \{ 1 - e^{-(k_a * C + k_d) * t} \} \dots\dots\dots(2)$$

where  $R(t)$  is the response at time  $t$ ,  $R_{eq}$  is the steady state response level,  $k_a$  is the association rate constant (units:  $M^{-1}s^{-1}$ ),  $k_d$  is the dissociation rate constant and  $C$  is the concentration of the injected peptide analyte. An offset was added to account for the refractive index differences between the analyte and the running buffer.

**Page 21, Paragraph starting on Line 1**

~~Data~~ Data for both  $k_a$  and  $k_d$  are quite consistent over the entire range of concentrations used. The difference between experimental and calculated data for both EL CD28 and RI CD28 peptide mimetics ~~stimated~~ estimated by  $\chi^2$  value is low being close to the noise level of the instrument. A linear regression plot of the rate of change in the response against response units was plotted using these values of  $k_a$  and  $k_d$  for each CD28 peptide analyte. The slope of this plot was then plotted against the concentration of the peptide to yield a  $K_d$  of  $2.44\mu M$ ,  $2.34\mu M$  and  $2.53\mu M$  for L CD28, EL CD28 and RI CD28 peptide mimetics, respectively, for binding to CD80-Ig. Consistent with the lack of PP II helix formation as observed by CD studies, a synthetic consisting of the hexapeptide motif alone did not bind CD80-Ig (data not shown).

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The average value of dissociation constant for RI CD28 peptide ~~mimetic~~ mimetic binding to CD80-Ig ( $K_d = 1.75\mu\text{M} \pm 0.67$ ) is a little lower than the value obtained with direct binding of the peptide to CD80-Ig ( $K_d = 2.53\mu\text{M}$ ). The values of dissociation constant obtained with varying concentrations of RI CD28 peptide ~~mimetics~~ mimetics were more consistent (Table 3).

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The ~~in-vitro~~ in vitro effect of varying concentration of the CD 28 peptide mimetics on CD4<sup>+</sup> lymph node cells and spleen cells from transgenic mice bearing the V $\alpha$ 4 V $\beta$  8.2 TCR specific for MBP Ac 1-11 was determined.

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~~Fig~~ Figure 5 shows a significant decrease in the proliferative responses of CD4<sup>+</sup> LNC and spleen cells to MBP Ac1-11 when treated with EL CD28 or RI CD28 peptide mimetics, and the effect is not dose dependent. Maximum inhibition was observed in CD4<sup>+</sup> LNC at 120 $\mu\text{M}$  concentrations of L CD28 (59.5%) followed by EL CD28 (47.6%) and RICD28 (45.7%) peptide mimetics. The proliferative responses of CD4<sup>+</sup> splenocytes were also decreased but to a lesser extent with the observed maximum inhibition of 50.2%, 38.2% and 42% with L CD28, EL CD28 and RI CD28 peptide mimetics respectively. A similar decrease in the proliferative responses to MBP was also observed (data not shown).

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The control RL CD28 and D CD28 peptides did not show inhibition. The hexapeptide consisting of the hydrophobic motif only did not show inhibition of T cell-proliferation. The hexapeptide consisting of the hydrophobic motif only did not show inhibition of T cell-proliferation. These results demonstrate that treatment with synthetic CD 28 peptide effectively blocked the expansion of encephalitogenic T cells *in vitro* suggesting the feasibility of a therapeutic application for these peptides ~~in-vivo~~ in vivo.

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~~Fig~~ Figure 6 shows that MBP stimulated CD4+ LNC bearing V $\alpha$ 4 V $\beta$  8.2 TCR exhibited significantly reduced frequency of IL-2 secreting cells in the presence of 50mM EL CD28 or RI CD28 peptide mimetics when compared to untreated cells. There was an increase in the frequency of IL-2 secreting antigen stimulated CD4+ LNC when higher concentrations of CD28 peptides were used. This observation perhaps reflects induction of apoptosis, since IL-2 is also known to sensitize activated T cells to cell death (50).

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~~Fig~~ Figure 7a is a representative histogram of three different experiments showing increased apoptosis of the CD4+ LNC cultured in the presence of indicated CD28 APR. Control cells cultured in the absence of antigen were 80% apoptotic (data not shown). These results suggest that the synthetic CD28 effectively competes with cell surface CD28 for binding B7-ligands on the APC and blocks the costimulation required for sustained survival of antigen stimulated T-cells.

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The biological activity of synthetic CD28 peptide analogues during antigen priming ~~in vivo~~ in vivo in EAE was then shown. B10.PL mice immunized with GP-MBP (200  $\mu$ g) in CFA were either left untreated or injected intravenously 500 $\mu$ g of EL CD28 or RI CD28 peptide mimetics or LCD28, RLCD28 and DCD28 peptides on the day of immunization. The vehicle treated mice and mice treated with: LCD28, RL CD28 and D CD28 peptide had maximum disease incidence of 100%, 100%, 91.5% and 91.5% and maximum mean cumulative score per day of 1.9, 1.8, 1.7 and 2 respectively. In contrast, significant inhibition of EAE was observed with mean maximum incidence of 70% and 60% and a maximum mean cumulative score of 1.1 and 0.74 in mice treated with EL CD28 and RI CD28 peptide ~~mimetics~~ mimetics, respectively. The effect of RI CD28 peptide injection lasted for the duration of observation (37 days) in one experiment.

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**Example 4. Treatment with Synthetic CD28 Peptide Mimetics Decreased IL-2 Production by Encephalitogenic T cells ~~*In Vivo*~~ *In Vivo*.**

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MBP reactive T cells that induce EAE are known to display a Th<sub>1</sub> phenotype secreting the ~~proinflammatory~~ proinflammatory cytokines IL-2, INF- $\gamma$  and TNF- $\beta$  (36. Kay, B.K., M.P. Williamson, and M. Sudol. 2000. *Faseb J* 14, no. 2:231). ELISPOT assay was used to assess *in vitro* cytokine production by draining lymph node cells and splenocytes upon restimulation from *in vivo* MBP-primed T cells. Mice were treated as described in Example 3, on day 0 of immunization. The frequency of IL-2 secreting lymph node cells decreased significantly EL-CD28 (2776  $\pm$  53.5;  $p < 0.05$ ) and RI CD28 (1753  $\pm$  37.7;  $p < 0.01$ ) treated mice as compared to vehicle (4846  $\pm$  14.3) and control RL-CD28 (3386  $\pm$  23.3) or D CD28 (2675  $\pm$  14.2) peptide treated mice.